Influencing hyperactive T cells by proteolytic enzymes

The present invention relates to a method for influencing hyperactive T cells by proteolytic enzymes and to the use of proteolytic enzymes for influencing hyperactive T cells.

With specific infections or cancer diseases or also renal diseases, a shift is observed in the homeostatic state of the various subpopulations of lymphocytes, granulocytes and monocytes. Normally, the organism is capable of re-establishing the distribution equilibria of the subpopulations during convalescence.

With chronic diseases, in particular, autoimmune diseases and the accompanying permanent stress on the immune system (e.g. by immune complexes, and in connection with chronically persisting viral infections such as AIDS), a shift in both the equilibria of the cell populations and the expression density of some specific antigens is observed in the course of the disease, which shift can no longer be compensated by the organism. This is in general described as an uncontrolled activation of the immune system. Animal models are available for studying such autoimmune diseases; for instance, the induction of allergic encephalomyelitis in mice serves as a model for multiple sclerosis, wherein after the administration of encephalic substance or specific peptides the mice show an autoimmune reaction which can be measured as paralysis in individual body parts.

Recently, a number of studies have been carried out that seem to suggest that the development of the above-mentioned diseases largely depends on the activation and expansion of specific T lymphocytes. Even in the case of those renal diseases that are substantially mediated by antibodies, a definite participation of the T cells could be detected.

In tests in which animals were immunized with antigens, it could be detected that specific T cells were activated and released cytokines.

Cytokines are important metabolic substances in the induction of various states of diseases. These are small proteins with molecular weights from 8000 to 30000, with each cytokine having its own amino acid sequence and cell surface receptors. They are produced by a multitude of different cell types and act on almost any tissue and organic system. The names given to the various cytokines do not follow any logic order, but rather take into account the biological characteristics thereof. IL-1 (interleukin-1) and TNF (tumor necrosis factor) are primary pro-inflammatory cytokines, and these two cytokines further act in a synergistic way in the induction of inflammations, shock and death.

The above-described immune reaction of the T cells with release of cytokines may manifest itself e.g. as multiple sclerosis, type I diabetes, rheumatoid arthritis or glomerulonephritis, or also as a transplant rejection.

The assumption that the modulation of T-cell surface molecules of the immune system may represent a special field of medication and that hyperactive T cells can be influenced thereby is supported by former knowledge.

Therefore, it has been the object of the present invention to provide a method for influencing hyperactive T cells.

This object is achieved by the invention indicated in claim 1. Advantageous developments are indicated in the subclaims.

According to claim 1 it is intended to use at least one proteolytic enzyme and, optionally, rutoside, for influencing hyperactive T cells. The occurrence of hyperactive T cells is characteristic of many immune system-mediated inflammation diseases, such as the above-mentioned diseases. Therefore, an improvement of the corresponding symptoms of the diseases until their complete abatement is possible through the use of proteolytic enzymes.

The influencing of hyperactive T cells by proteases presumably functions in the following way:

One of the proteases, for example trypsin, is subjected to an enteropancreatic recirculation in the body. After it has been secreted by the exocrine pancreas, it is resorbed in the digestive tract lumen and transported into the blood, from where it is resecreted into the pancreas. Trypsin is a standard constituent of the serum. About 10% of orally administered trypsin finally ends in the blood. Like other serum proteases, serum trypsin is bound to an antiprotease, the α2-macroglublin. In this complex trypsin is protected from autodigestion and also from decomposition by other serum proteases. It maintains, however, its enzymatic activity. At places of inflammation where the vascular permeability is locally enhanced, serum protein enters into the interstitial region. Trypsin is thereby enriched at the place of inflammation.

It is assumed that trypsin modifies, in particular, the cell surface molecules CD4, CD44 and B7-1, which are important regulators of the T-cell immune reaction. In contrast to its action in the digestive tract, where trypsin can decompose a wide range of substances, its effect seems to be limited only to a few surface molecules, among others, the three that have just been mentioned. This might e.g. be due to the fact that in an inflamed tissue the binding sites for the trypsin are masked by glycosylation or are hidden in the protein core.

Each of the above-mentioned three cell surface molecules CD4, CD44 and B7-1 participates in the regulation of the limit value for a T cell activation. T cells only attack the antigen which is offered to them on antigen-presenting cells. In the course of this recognition process the antigen receptor of the T cell (TCR) specifically binds to a major histocompatibility complex (MHC complex) and to a specific peptide fragment of the native antigen. This cooperation defines the specifity of T cell recognition. There is a limit value at which, when the same is exceeded, T cells are activated. The definition for the conditions under which T cell activation takes place, i.e. the minimal signal strength needed by a T cell for surpassing the limit value for its activation, is as follows:

T cell activation takes place whenever a minimal number of bonds (ligations) of the T cell receptor to an MHC complex and antigen is exceeded within a defined period of time. This number is assumed to be constant for T cells and may e.g. be in the order of 100 TCR ligations per cell and second. This number (X) defines the T-cell activation limit value. According to the current definition T cells with a high affinity (T cells the TCRs of which show a high affinity for a given MHC peptide complex) need fewer ligands (MHC: nominal peptide complexes) for the activation than T cells having a low affinity for the antigen.

By contrast, it is assumed in the present invention that the T-cell activation limit value is not a rigid intrinsic feature of the T cells but, in addition, a function of the activation stage of the T cell and the accompanying expression of co-stimulating molecules. Thus the number of T-cell receptor ligations needed for the activation is a function of the preceding interactions between T cells and antigen, namely period of time since the last interaction, strength and frequency of the interaction with antigen, and the interaction of special cytokines or other mediators that additionally stimulate the cells. Native T cells which have not yet interacted with an antigen express fewer accessory molecules and need

more antigens to be stimulated than do normal resting or quiescent T memory cells (Pihlgren et al., J. Exp. Med., 184: 2141, 1996). This means that when the number X is assumed to stand for TCR ligations per second for the activation of native cells, this number should be < X for normal memory cells. These memory cells have been preactivated by former interactions with antigens, in which process the antigen was secreted from the organism over time and the cells returned into a more quiescent stage. They maintain, however, a higher expression of accessory molecules on their surface and need smaller antigen concentrations for their activation.

According to the model underlying this invention, hyperactive T cells are those cells that carry relatively many accessory molecules on their cell surface and are continuously stimulated by the interaction of their specific T cell receptors with antigens, e.g. autoreactive, tumor antigen-specific, transplant-specific, allergen-specific or virus-specific T cells which can be observed in autoimmune diseases, cancer, transplantations, allergies or chronic viral infections. It is true for hyperactive T cells that these already react to less than 1/3 of the amount of antigen required for inducing a reaction in a "normal", i.e. non-hyperactive, T cell. This T cell reaction is characterized by the production of cytokines and chemokines and by proliferation or cell-mediated cytolysis.

It is presupposed for the present invention that hyperactive T cells need even fewer TCR ligations than do normal memory cells for a renewed activation, i.e. their activation limit value is <<X, because hyperactive T cells express more accessory molecules on their surface than do normal memory cells. The expression of accessory molecules is here proportional to the preactivation stage of the T cells (number of accessory molecules: quiescent/native T cells < memory/activated T cells < memory/hyperactivated T cells). Furthermore, the number of TCR ligations required for T cell activation is inversely proportional to the number of expressed accessory molecules (X: quiescent T cells < memory cells < hyperactivated T cells). Since the enhanced reactivity of hyperactive T

cells results from their enhanced expression of accessory molecules, the cleavage or neutralization of this surplus of accessory molecules with the help of enzymes, such as trypsin, can adapt the hyperactive T cells in functional terms to the normal memory T cells. In the case of an extensive neutralization of the accessory molecules hyperactive T cells can functionally be adapted even to native T cells. After the activation limit value for hyperactive T cells has been raised by way of an enzyme treatment, the dose of existing antigen (MHC/peptide complex density on antigen-presenting cells) that was sufficient for obtaining T cells in the hyperactive stage should fall below the limit value for renewed T cell activation, whereby the T cells are enabled to return from an hyperactivated stage into a more quiescent stage. Thus the T cell-mediated symptoms should decline or at least be influenced in a favorable manner.

The other proteases act in a similar way as trypsin.

In a preferred embodiment, trypsin, bromelain, papain and, optionally, rutin are used as the proteolytic enzyme, or a combination thereof.

The enzymes used according to the invention can be isolated in an inexpensive way from the following raw materials.

Bromelain is a proteolytically active enzyme from squeezed pineapple juice and can also be isolated from ripe fruits.

Papain is a proteolytic enzyme obtained from the latix of unripe, fleshy fruits of the melon tree Carica papaya. Pure papain is a crystalline polypeptide with a molecular weight of 23,350 and consists of a chain of 212 amino-acid residues with 4 disulfide bridges; sequence and spatial structure are known. Papain has many applications: Thanks to its protein-cleaving property it is used as a "meat tenderizer", for clarifying beer, for making

bread or hard biscuits, in leather preparation, in the textile industry for boiling off silk and for preventing wool matting, in the tobacco industry for quality improvement, and for recovering silver from used photographic material, further in bacteriology for peptone isolation. In the medical field papain already serves to promote enzymatic digestion, it serves enzymatic wound cleaning or as an additive to cleaning agents for dental prostheses. For special purposes papain preparations are also offered bound to carriers such as plastic polymers or agarose. Papain has also been used as a catalyst for the synthesis of oligopeptides.

Trypsin is a proteolytic enzyme which is also formed in the pancreas and is already used therapeutically in combination with other enzymes. It belongs to the serine proteases. Crystalline trypsin has a molecular weight of about 23,300, it is soluble in water, but not in alcohol, it has an optimum effect at pH values of 7 to 9 and cleaves peptide chains specifically on the carboxyl terminal side of the basic amino-acid residues L-lysine and L-arginine. The spatial structure of trypsin, which consists of 223 amino acids, is known.

A particularly high efficiency is observed when a combination of the enzymes bromelain, papain and/or trypsin is used. Apart from the remarkable and unexpected effect of these enzymes, the combined use of said enzymes offers the further advantage that harmful side effects are not observed even in the case of long-term application.

Furthermore, rutoside may be admixed to the drug in addition. Rutoside is a glycoside belonging to the flavonoids.

A particularly high efficiency is achieved by the combined use of 20 to 100 mg bromelain, 40 to 120 mg papain and 10 to 50 mg trypsin per dose unit.

Quite particularly preferred is a combination of 90 mg bromelain, 120 mg papain and

100 mg rutoside x $3H_2O$.

In another preferred embodiment a combination of 48 mg trypsin, 90 mg bromelain and 100 mg rutoside x $3H_2O$ is used. This combination is e.g. sold under the name Phlogenzym by the company Mucos Pharma GmbH & Co. in Germany.

In a further preferred embodiment use is made of 10 to 100 mg, particularly preferably 100 mg rutoside x 3 H_20 per dose unit.

The drug may further contain all of the standard adjuvants and/or vehicles.

For instance lactose, magnesium stearate, stearic acid, talcum, methacrylic acid, copolymer type A, Shellack, Makrogol 6000, dibutyl phthalate, vanillin, titanium dioxide, white clay, polyindone, yellow wax and Carnauba wax are possible as adjuvants and vehicles.

The invention further relates to the additional use of α_2-macroglobulin (α_2-M). α_2-M is one of the most important inhibitors of proteinases. α_2-M reacts with a multitude of endopeptidases. The reaction of α_2-M with the respective proteinase normally takes place such that α_2-M is subjected to a conformation change after it has come into contact with the proteinase and then retains the same in the molecule. The enzyme inhibition is due to steric obstruction - though the active center of the enzyme maintains its function. Due to the influence on the proteinase α_2-M contributes to an immunomodulation by influencing T cells.

Furthermore, the invention relates to a method for influencing hyperactive T cells, wherein the T cells are contacted with one or several proteolytic enzymes and, optionally, with rutoside.

The following figures, tables and examples shall explain the invention in detail.

Fig. 1 CD4 (epitope Leu3a) modulation by proteases

Indicated is the median of the relative fluorescence intensity of the target cells (lymphocytes). The positive control (reference) are untreated cells which were incubated with the CD4-specific monoclonal antibody. The negative control are untreated cells which were incubated with the antibody isotype. The incubation time with the enzymes was 60 min. Shown are the data of donor 2 (cf. Table 1) as the mean value of duplicate determinations and standard deviation.

Fig. 2 CD4 (epitope OKT4 and Leu3a) modulation by trypsin

Indicated is the median of the relative fluorescence intensity of the target cells (lymphocytes). The positive control (reference) are untreated cells which were incubated with the CD4-specific monoclonal antibody. The negative control are untreated cells which were incubated with the antibody isotype. The incubation time with the enzymes was 60 min. Shown are the data of a donor (cf. Table 2) as the mean value of duplicate determinations and standard deviation.

Fig. 3 Expression of surface molecules after Phlogenzym treatment in vitro.

The T-cell hybrid clone F.10.9 (upper figures) and the B-cell tumor clone B220 (lower figures) were each incubated with 50 µg/ml of Phlogenzym at 37°C and subsequently labeled.

a: label of the isotype control

b: label of HEL-treated control cells

c: label after Phlogenzym treatment

Fig. 4 Proteolipid protein (PLP):139-151 peptide-specific proliferative "recall response" in mice which were fed with Phlogenzym or HEL

The mice were immunized with PLP:139-151 peptide and fed with 41 mg of Phlogenzym (filled symbols) or hen egg lysozyme (HEL; blank symbols). On day 30 the splenic cells were analyzed in the presence of the indicated PLP peptide concentrations for their proliferative "recall response". The data are represented as average values with standard deviation of three parallel cultures of a mouse of each group and are representative of five mice tested in each group. These results were reproduced in two experiments.

Fig. 5 Cytokine "recall response" in mice which were treated with Phlogenzym and HEL as the control

5 SJL mice per group were immunized with PLP:139-151 peptide. From the day of their immunization they were artificially fed twice a day with 42 mg of Phlogenzym or an equivalent dose of HEL. Lymphoid cells were assayed in ELISA spot assays for IL-5 (A) and IL-4 (B) production in the presence of medium alone or PLP:139-151 peptide at the indicated concentrations.

White bars: diseased control mice fed with 42 mg of HEL, analyzed on day 21
Gray bars: non-diseased mice fed with 42 mg of Phlogenzym, analyzed on day 21
Black bars: HEL-treated mice tested on day 60 after reconvalescence from allergic encephalomyelitis.

The data show the average value and standard deviation of three parallel cultures of a mouse of each group and are representative of five mice which were tested in this experiment in each group. The results were reproduced in three experiments.

Fig. 6: Development of allergic encephalomyelitis in mice which were orally treated with Phlogenzym and HEL.

Active allergic encephalomyelitis was injected into groups of 10 SJL mice each per experiment by PLP:139-151-peptide immunization while the mice were artificially fed twice a day with 8.2 mg (black circles, 20 mice) or 41 mg (bright circles, 30 mice) of Phlogenzym from the first day of immunization. Control mice (also 10 mice per experiment) were fed with equivalent amounts of HEL (bright squares). Since there was no difference between the groups fed with 8.2 mg Phlogenzym and those fed with 41 mg HEL, the data were calculated for all of the total of 50 control mice. The average disease rate was 3.7 for the control mice and 1.6 for the mice treated with 8.2 mg Phlogenzym.

Example 1

Material and methods

1. Material

Cell culture:

The cell culture medium RPMI 1640 was used with the following addition: $NaHCO_3$ (Biochrom, 2 g/1); L-glutamine (Biochrom, 2mM), Na pyruvate (Biochrom, 1 mM), NaN_3 (Sigma, 0.01%).

In the case of a stimulation of the cells either phythemagglutinin M (Biochrom, 5 µg/ml) or γ-interferon (100 U/ml) was used. The cells were stimulated for 1 to 3 days under addition of 10% fetal calf serum (Biochrom).

Freshly isolated, human, peripheral, mononuclear blood cells (citrate blood) were used as targets for the enzymes to be analyzed. After the standard isolation by means of Ficoll the cells were washed several times and freshly used in the experiments.

In vivo experiments:

The T cell clone F.10.9 and the B cell clone B220 were used for in vitro experiments with Phlogenzym. In in-vivo experiments with Phlogenzym (Mucos Pharma GmbH & Co. GmbH) and hen egg lysozyme (HEL; Sigma, St. Louis, MO) as the control, female SJL mice (The Jackson Laboratories) at the age of 6 to 10 weeks were used.

The peptide proteolipid protein (PLP):139-151 (H-KISQAVHAAHAE-OH; Princecton Biomolecules, Columbus, OH) used for inducing allergic encephalomyelitis in mice was mixed with Complete Freud's Adjuvans (CFA; 2.5 mg/ml M tuberculosis H37RA; Difco Laboratories, Detroit, MI) in Incomplete Freud's Adjuvans (IFA; Gibco BRL, Grand Island, NY) to obtain an emulsion of 1 mg/ml with which each mouse was subcutaneously injected with 50 µl (50 µg/mouse). From day 8 after immunization the mice were examined for the development of paralytic symptoms and the degree of severity of the disease was measured according to the standard scale.

Degree 1: relaxed tail
Degree 2: weakness in the hind legs
Degree 3: complete paralysis of the hind legs
Degree 4: quadriplegia

Degree 5: death

To ensure feeding of the paralyzed mice, long water tubes were inserted into the cages and solid food was put into the nest.

2. Proliferation test

This test was carried out as in Lehmann et al., Nature, 1992, 358, 155-157. Single-cell suspensions were prepared from a splenic organ and 1 x 10^6 of these cells were sown out in a respective well of a 96-hole flat-bottom microtiter plate in serum-free HL-1 medium (Bio Whittaker, Walkersville, MD), supplemented with 1 mM L-glutamine. Antigens or peptides were added to obtain a final concentration of 100 µg/ml. During the last 18 hours of the 4-day culture ^{3}H thymidine (1 µCi/well) was added. The incorporation of the radioactive label was measured by scintillation counting.

3. Cytokine measurement by ELISA spot tests

The method has already been described by Forsthuber et al., Science (1996), 271, 1728-1730.

ImmunoSpot plates (Autoimmun Diagnostika, Beltsville, MD) were coated overnight in PBS with the antibodies R46-A2(4 µg/ml) or TRFJS- (5 µg/ml) that specifically bind IFN-γ and IL-5, respectively. The plates were unspecifically blocked with 1% BSA in PBS at room temperature for 1 h and washed with PBS four times. 1 x 10^6 splenic cells were solely plated in HL-1 medium or with peptides at specific concentrations and cultured in the case of IFN-γ for 24 h and in the case of IL-5 for 48 h. Subsequently, the cells were removed by washing and the antibody was added for detection (1 µg/ml XMG1.2-HRP for IFN-γ or 4 µg/ml TRFK4 for IL-5) and incubated overnight.

For IL-5 the antigen anti-IgG2a-HRP (Zymed) was added and incubated for 2 h. The last antibody bound to the plate was visualized by adding AEC. For the assessment of the results a Series 1 ImmunoSpot Image Analyzer (Autoimmun Diagnostika) was used.

4. Monoclonal antibodies used

Monoclonal antibodies were used for the specific detection of the surface structures of the leukocytes. On the corresponding antigens these antibodies respectively detect a defined epitope which in the analyzed antigens occurs in the structure only once. Survey 1 shows the analyzed surface labels, the monoclonal antibodies, as well as the analyzed target cells.

Survey 1: Analyzed surface label, monoclonal antibodies used, and target cells of the enzyme treatment

Label	Epitope – antibody designation	Fluorochromium	Producer	Target cell
CD4	Leu3a OKT4	PE FITC[1]	B.D. Ortho[2]	T lymphocytes T lymphocytes

[1] Fluorescein isothiocyanate, green fluorescence;

[2] Ortho Diagnostics

5. Incubation Conditions

The freshly isolated and prepared cells were incubated with the enzymes bromelain, papain and trypsin (drug ingredients of the company Mucos Pharma GmbH & Co.) or with Phlogenzym (sold by the company Mucos Pharma GmbH & Co.) and HEL (hen egg lysozyme) at the concentrations indicated in the respective legends of the table and figure. In the mixture of the three enzymes the mixing ratio was 22.7 : 15.5 : 11.9 (bromelain : papain : trypsin, based on 40 µg/ml, "BPT"). Three enzyme concentrations (40, 10, 2.5 µg/ml) were analyzed for BPT and a concentration of 50 µg/ml of Phlogenzym was used. Incubation took place in a serum-free medium at 37°C.

The proteases were directly prepared before incubation. 0.01% sodium azide was contained in the cell culture media. The cells are inhibited by this addition to express receptor molecules again during the incubation process or the washing operations. After the cell culture medium has been washed, the cells can be activated again (not demonstrated).

After a corresponding incubation of the cells with the respective enzymes, washing of the enzymes and labeling with monoclonal antibodies (according to manufacturers' instructions) the surface labels were immediately analyzed.

6. Analytical flow cytometry

All analyses regarding the modulation of cell surface molecules were carried out by means of analytical flow cytometry (FACSCAN, company Becton Dickinson, Heidelberg) using a device-specific software (Lysis I). The Cell Quest Software (Becton Dickinson, Mountain View, CA) was used for experiments with Phlogenzym. With a corresponding

setting of the device and the simultaneous use of references, i.e. cells treated without enzymes but used in the same procedure, the measurement was carried out.

For the monoclonal antibody a corresponding fluorescence-conjugated isotype control was used. This is murine immunoglobulin with which the capacity of the unspecific binding of the target cells is determined by flow cytometry.

10,000 cells were measured per histogram. The respective cell population was separated with a so-called "electronic gate" which subsequently contained at least 3,500 cells.

7. Illustration of the results

7.1 Proteolytic cleavage of surface molecules by trypsin, bromelain, papain and BPT

The data were evaluated and analyzed independently of the measuring operation on the flow cytometer by means of a device-specific software. The respective histograms of the controls, i.e. the untreated cell samples, were compared with the histograms of the enzyme-treated cells.

The rough illustration is an optically impressive, but relatively unclear histogram in which various individual measurements are shown in superimposed form. As for these analyses, it is not the percentage of a subpopulation in the total leukocyte population that is the measured value, but the relative receptor density represented as fluorescence intensity.

Data that reflect the relative fluorescence intensity of the individual measurement can be derived from these histograms. This is a measure of the relative receptor or surface molecule density in a measured cell population.

The column diagrams are logarithmic illustrations of the median of relative fluorescence (Figs. 1, 2). The reduction of the density of the respective surface molecule in response to the enzyme concentration can thereby be represented in comparison with the reference.

The tables contain the data of independent experiments (Tables 1, 2). For instance, when a value of 40% is indicated in the table, this means that in this antigen 40% of all surface molecules are changed by the enzyme such that the specific monoclonal antibody no longer recognizes its epitope. If no reduction is observed, the value "0" appears in the tables. The indicated percentage thus expresses the enzyme activity with respect to the individual antigens. Values up to 20% are considered to be of no relevance in the individual case.

For an evaluation of the effect of the enzymes on the individual antigens, it is of advantage to select a suitable reference standard. With a few exceptions, all of the analyses were carried out under standardized incubation conditions. The half-effect concentration known from former research reports can thus be indicated for these experimental conditions.

For the calculation of the half-effect concentration the data were evaluated by means of non-linear regression. The median of fluorescence intensity versus used enzyme concentration and reference are related with one another, and the amount of enzyme which leads to a 50% reduction of the relative fluorescence intensity, as well as the receptor density changed in the structure can be calculated therefrom.

Table 1 illustrates the CD4 (epitope Leu3a) modulation on lymphocytes by the proteases bromelain, papain, trypsin and the protease mixture BPT. Indicated is the reduction in percent of the median of relative fluorescence intensity, which is a measure of the enzyme activity. The incubation time with the enzymes was 60 min. The results of two experiments with cells from two different donors are shown.

Enzyme	No.	40 µg/ml	10 µg/ml	2.5 µg/ml
Bromelain	1	24.6	0	0
	2	16.2	0	0
Papain	1	2.5	3.2	0
	2	0	0	5.7
Trypsin	1	99.3	93.0	64.1
	2	99.4	96.2	57.5
BPT	1	98.3	49.3	23.0
	2	99.4	79.2	20.2

Table 2 shows the modulation of the CD4 epitope Leu3a and OKT4 by trypsin on lymphocytes. Indicated is the reduction in percent of the median of relative fluorescence intensity, which is a measure of the enzyme activity. The incubation time with the enzymes was 60 min. The data of one donor are shown.

Enzyme	Epitope	40 µg/ml	20 µg/ml	10 µg/ml
Trypsin	Leu3a	98.5	96.7	87.1
	OKT4	6.5	6.3	19.5
	Epitope	5 µg/ml	2.5 µg/ml	1.25 µg/ml
Trypsin	Leu3a	65.2	41.9	28.8
	OKT4	13.3	10.8	9.3

7.2 Proteolytic cleavage of surface molecules by Phlogenzym

An analysis was carried out as to which cell surface molecules participating in the interaction between T cells and antigen-presenting cells (APZ) can be cleaved off by Phlogenzym. Fig. 3 shows that an enzyme treatment (50 µg/ml) of T and B cell hybridomas for two hours considerably reduces the detection of CD4, CD44 and B7-1 by adequate FACS labels whereas the CD3 label is not affected. Likewise, the detection of MHC class I, MHC class II and LFA-1 molecules after incubation with Phlogenzym was not changed, whereas the detection of ICAM-I and B7-1 cell surface molecules was slightly reduced. These results demonstrate that, despite their ubiquitary cleavage motifs, proteases can create very selective changes in the cell surface molecules.

7.3 Shift to the right in the dose-effect curve for T cell activation in mice which had Phlogenzym administered in oral form.

Since the threshold value for T cell activation depends on the number of accessory molecules, the cleavage of CD4, CD44 and B7-1 molecules should create a shift to the

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right in the dose-effect curve for the T cell response. Therefore the proliferative "recall response" to peptide PLP:193-151 was tested in freshly isolated splenic cells in mice which were immunized with this peptide and were artificially fed with Phlogenzym or HEL via a tube (41 mg enzyme/mouse, twice a day). The results are shown in Fig. 4. The response of the Phlogenzym-treated mice was about 10-40% of the response that was measured in control mice. In addition, the dose-effect curve in the Phlogenzym-treated group was shifted to the right at submaximal peptide concentrations. This demonstrates that a Phlogenzym treatment in vivo modifies the T cell function in a way corresponding to an increased threshold value for T cell activation which results from the cleavage of accessory molecules that take part in the T-cell/APZ interaction.

7.4 $T_{helper}2$ cell switching of the autoantigenic specific T cell response in Phlogenzym-treated mice

A weak T cell activation with low avidity normally induces a $T_{helper}2$ cell response (Th2). Since B7-1 molecules are conducive to Th1 development, the cleavage of said molecules might entail a Th2 switch of the autoimmune T cell response. To check this possibility, mice were immunized with the peptide PLP:139-151, and the cytokine quality of the "recall response" to this peptide was examined. The mice were fed twice either with 41 mg/mouse/day of Phlogenzym or with HEL and tested 21 days after immunization; at a time when most of the HEL-fed control mice, but none of the Phlogenzym-treated mice, had developed allergic encephalomyelitis. Splenic cells of the diseased HEL-fed mice showed no significant IL-4 (Fig. 5A) or IL-5 (Fig. 5B) production in response to the PLP:139-151 peptide in comparison with the medium control (white bars), but showed a strong IFN-γ "recall response" to this peptide. The Phlogenzym-treated mice which were not diseased showed the same strong PLP:139-151-specific IL-4 and IL-5 production (gray bars). The IFN-γ "recall response" was not significantly different between the two groups. Therefore, the Th2 response was intensified in the Phlogenzym-treated mice.

This cytokine "recall response" of the Th2 type was also observed in the control mice three months after immunization; at a time at which the mice had recovered from allergic encephalomyelitis (black bars).

7.5 Oral administration of hydrolytic enzymes prevents the development of allergic encephalomyelitis

Every mouse was artificially fed with 8.2 mg Phlogenzym via a tube twice a day, which after weight correction corresponds to the equivalent human dose. The same amount of HEL was administered to the control mice. Together with the enzyme administration, immunization with the disease-inducing PLP peptide 139-151 took place and was continued until the end of the experiment. The mice were examined daily as to whether they developed paralytic symptoms characteristic of allergic encephalomyelitis. 39 of the 50 control mice (78%; Fig. 6; rectangular symbols) developed symptoms with an average disease rate of 3.7, whereas only 4 of the 20 Phlogenzym-treated mice (20%; Fig. 6; circular symbols) developed symptoms with an average disease rate of 1.6. Since mice have a faster metabolism than humans, the Phlogenzym dose was increased to test whether this increase could create a stronger effect. It was found that 5 times the dose (41 mg/mouse, twice a day) completely inhibited the development of allergic encephalomyelitis in a total of 30 mice tested (Fig. 6; triangular symbols). With a continuous treatment the mice showed no symptoms during the one-month observation period, from which the conclusion was drawn that the oral administration of Phlogenzym has a strong disease-inhibiting effect.